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TITLE: Differential Processing of Cyclin E Variants in Normal vs Tumor Cells and their Role in Breast Cancer Oncogenesis

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Annual Summary-From 9/01/99 to 8/31/00

Training grant #DAMD17-99-1-9214

Title: Differential Processing of Cyclin E variants in normal vs. tumor cells and their role in breast cancer oncogenesis.

Research Accomplishment:

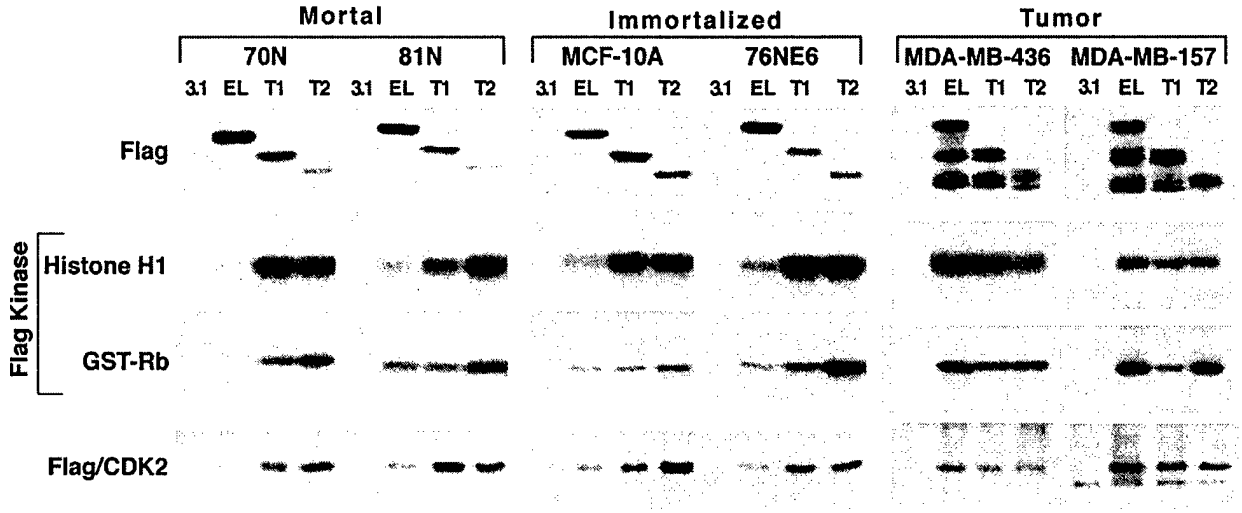
During the 10 months of this award period we were successful in fulfilling the several components of Task 1. The major goal of this fellowship is to examine the difference in expression of lower molecular weight isoforms of cyclin E between normal and tumor cells. To this end we had proposed to determine the differences in processing of the full length cyclin E into its LMW forms between normal and tumor cells. As a first step toward fulfillment of this aim we had proposed to repeat transfection of FLAG-tagged cyclin E between normal and tumor cells using a GFP reporter gene transfection. We also had proposed to repeat the transfection in 2 other normal and cell lines. We had further proposed to perform immunoprecipitation study of the truncated FLAG cyclin E on transfected cells and lastly to further clone more truncated cyclin E forms within the proposed region for processing. These tasks are now complete, the results presented as figure 1 and described below:

To examine the biochemical activity of the LMW forms of cyclin E as compared to the full length form we used 3 different constructs of cyclin E that are FLAG tagged at the C-terminus in transfection assays using normal and tumor cells. The 3 constructs are cyclin EL-FLAG, Trunk 1 and Trunk 2. Trunk 1 and 2 bracket only the LMW isoforms of cyclin E and not the full length form, while cyclin E-L represents the full-length form. Trunk 1 initiates at AA40 and brackets EL2-6 and Trunk 2 initiates at AA65 and brackets EL-5 and EL-6. We have examined the biochemical activity associated with the protein products of cyclin EL, Trunk-1, and Trunk 2 constructs in several normal and tumor cell lines (Fig 1).

Transfection of the 2 normal mortal cell strains (70N and 81N) and the 2 immortalized cell lines (MCF-10A and 76NE6) with each of the 3 cyclin E-FLAG construct results predominantly in the expression of the full-length form of each construct as shown in the western blot analysis using FLAG antibody (Fig 1). Following transient transfection of each cell line with the cyclin E-FLAG constructs, the activity of the resultant protein products were assessed by Histone H1 and GST-Rb phosphorylation, and immune-complex formation with CDK2 (Fig 1). Histone H1 and GST-Rb were used as substrates for active cyclin E/CDK2 complexes in immunoprecipitates prepared with an antibody to FLAG. This analysis revealed that the truncated forms of cyclin E activate a greater amount of kinase activity than the EL form in the four normal cell lines (mortal or immortalized). In all 4 normal cells, Trunk 1 and Trunk 2 phosphorylated both Histone H1 and GST-Rb much more effectively than the full length cyclin E. The expression levels of the truncated forms are generally less than the EL form (Fig 3, lanes EL, T1, and T2 bands in the top panels). In spite of this lower protein expression, the kinase activity is significantly higher and this effect is more noticeable in the non-tumor cell lines. The same non-tumor cell lines also show an increased amount of CDK2 immunoprecipitating with the truncated cyclin E. Hence, the kinase activity associated with each construct was proportional to the amount of complex formation between FLAG constructs and CDK2 in each of the normal cell lines (compare Flag kinase panels with Flag/CDK2 panel). Although this could represent an increased affinity of the truncated forms of cyclin E for CDK2, it is more likely it involves an up regulation of CDK2 protein levels since the CDK2 levels increase after transfection when examined by western blot. Collectively, these results suggest that in normal cells the forced expression of the LMW forms of cyclin E have the ability to bind to and activate CDK2 in order to phosphorylate substrates such as Histone H1 and GST-Rb much more effectively than the full length form.

Tumor cells behave differently than normal cells in processing and activating these constructs. For example, transfection of the 2 tumor cell lines, MDA-MB-157, and MDA-MB-436, with the 3 constructs resulted in the processing of several LMW forms as well as the full-length form of each construct (Fig 1). Additionally the kinase activity associated with the full-length construct is higher than Trunk 1, which was in turn higher than Trunk2 in tumor cells. Lastly, immune-complex formation of the 3 cyclin E-FLAG constructs to CDK2 in tumor cells shows that

the full length cyclin E-FLAG construct binds more CDK2 than either Trunk1 or Trunk 2-FLAG constructs. Since tumor cells have the machinery to process the full length cyclin E into its LMW forms, the activity associated with the cyclin EL-FLAG is a combination of the full length and all the LMW forms it is processed to. However, unlike tumor cells, normal cells do not process cyclin EL-FLAG into its LMW forms. Hence, the forced expression of the LMW forms of cyclin E in normal cells provide us with an ideal model system to assess the biological activity of the individual protein products of Trunk-1 and Trunk 2 constructs as compared to the full length form.



The LMW forms of cyclin E are biochemically active. Cyclin E-L- and cyclin E-FLAG constructs Trunk 1 and Trunk 2 were transfected into 2 mortal cell strains (81N and 76N) 2 immortalized (MCF-10A and 76NE6), and 2 tumor cell lines (MDA-MB-157 and MDA-MB-436) harvested 16 hours post transfection and subjected to; Western blot analysis with anti-FLAG antibody, Histone H1 and GST-Rb kinase analysis, or Immune complex formation with CDK2. For Western blot analysis 50 μg of protein extract from each condition was analyzed with polyclonal antibody to FLAG. For kinase activity and immune complex formation, equal amounts of protein (250 μg) from cell lysates were prepared from each condition and immunoprecipitated with anti-FLAG antibody (polyclonal) coupled to protein A beads. Histone H1 and GST-Rb were used as substrates in the kinase reaction. For each condition we show the resulting autoradiogram of the histone H1 and GST-Rb SDS-PAGE. Immune complex formation with CDK2 was assessed by subjecting the anti-FLAG immuno-precipitates to Western blot analysis using a monoclonal antibody to CDK2.

No technical of unexpected difficulties were encountered and no deviations from the original Statement of Work were experienced nor anticipated for future years of the award.

Bulleted list of key research accomplishments:

1) Normal cells do not have the machinery to process cyclin E into its Lower Molecular Weight (LMW) Forms.
2) Tumor cells process cyclin E into its LMW Isoforms.
3) The LMW isoforms of cyclin E uniquely found in tumor cells are biochemically hyperactive as compared to the full length form.

List of Reportable Outcomes

Papers:

1. Harwell, R.M., Porter, D.C., Danes, C., and Keyomarsi, K. Processing of cyclin E differs between normal and tumor breast cells. Cancer Research 60: 481-489 (2000).

2. Porter, D.C., Zhang, N., Danes, C., McGahren, M.J., Harwell, R.H., Faruki, S., and Keyomarsi, K. Tumor-Specific Proteolytic Processing of Cyclin E Generates Hyperactive Lower-Molecular-Weight Forms. Mol. Cell. Bio., 21: 6254-6269 (2001).

Abstract:

Lavu, H., Harwell, R., Danes, C., and Keyomarsi, K. Clonal variants of MCF-10A cells have a deregulated cell cycle. Proceed. Amer. Assoc. Can. Res. Volume 41, #2317 (2000)